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Volume 3 of 3

STUDY TITLE

Myacide AS -Assay for bronopol and bronopol impurities by HPLC
(Test Method PM/01645/02e) prepared by
Mr. Thomas Schellenberger, May 4, 2012 and
British Pharmacopoeia (BP)
BP-2011 Method

BASF CORPORATION

MYACIDE AS
EPA Registration No. 33753-3

DATA REQUIREMENTS

US EPA Guideline Number OCSPP Series 830
OCSPP No. 830.1800
(Analytical Method)

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Total pages: 16

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA sec. 10(d)(1)(A),(B), or (C).

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The information in this volume is not required to meet the GLP requirements specific in 40 CFR Part 160.

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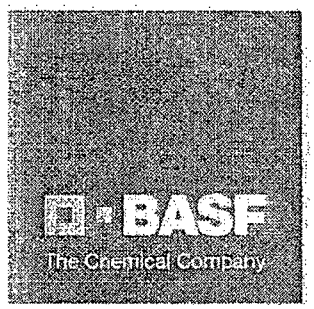
GKA Competence Center Analytics

Test Method PM/01645/02e



Title: **Assay for bronopol and bronopol impurities by HPLC**

Effective date: May 4th, 2012

Created by: Thomas Schellenberger

Released by Schellenberger, Thomas (SBTH)
(e-signed in ELAS)

implementation by Leyendecker, Michael (LEYM)
e-signed in ELAS)

Test Method PM/01645/02ee

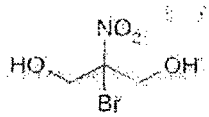
Assay for bronopol and bronopol impurities by HPLC

1 Keywords

1.1 Analytes:
1.1.1 2-Bromo-2-nitropropane-1,3-diol (bronopol)
1.1.2 Sodium bromide
1.1.3 Tris(hydroxymethyl)nitromethane
1.1.4 2-Methyl-2-nitropropane-1,3-diol
1.1.5 2-Nitroethanol

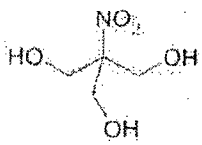
1.2 Matrix:
1.2.1 Bronopol brands

1.3 Structures:
1.3.1 2-Bromo-2-nitropropane-1,3-diol (bronopol)



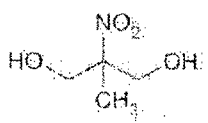
Mol. mass = 199.99 g/mol
Molecular formula $C_3H_6BrNO_4$

1.3.2 Tris(hydroxymethyl)nitromethane



Mol. mass = 151.12 g/mol
Molecular formula $C_4H_9NO_5$

1.3.3 2-Methyl-2-nitropropane-1,3-diol

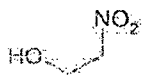


Mol. mass = 135.12 g/mol
Molecular formula $C_4H_9NO_4$

Test Method PM/01645/02ee

Assay for bronopol and bronopol impurities by HPLC

1.3.4 2-Nitroethanol



Mol. mass = 91.07 g/mol
Molecular formula $C_2H_5NO_3$

2 Principle

The samples and the bronopol standard are dissolved in a solution of acetamidophenol and diluted with the diluent. The other standards are dissolved directly in the diluent and diluted. The material is assayed by reversed-phase HPLC with gradient elution and ultraviolet detection at 214 nm. Quantitation is done by external standardization. All solutions are kept in amber-colored glassware.

3 Range

w(Bronopol)	= 60 g/100 g –	100 g/100 g
w(Sodium bromide)	= 0.01 g/100 g –	1 g/100 g
w(Tris(hydroxymethyl)nitromethane)	= 0.01 g/100 g –	1 g/100 g
w(2-Methyl-2-nitropropane-1,3-diol)	= 0.01 g/100 g –	1 g/100 g
w(2-Nitroethanol)	= 0.01 g/100 g –	1 g/100 g
w(Unknown impurities, quantitated as bronopol)	= 0.01 g/100 g –	1 g/100 g

(it is at approx. 200 mg of sample/50 ml with all impurities except for the major component bronopol; with bronopol it is approx. 20 mg of sample/50 ml).

The lower limit of the range for the impurities corresponds to the smallest concentration used in the calibration run. The range can be expanded to higher concentrations by using suitable calibration points.

4 Safety note

The method described involves the handling of hazardous substances. Attention is therefore drawn to the various provisions governing the handling of potentially dangerous materials. Protective measures of a technical, organizational and personal nature must be observed.

Test Method PM/01645/02ee

Assay for bronopol and bronopol impurities by HPLC

5 Reagents

5.1 3-Acetamidophenol (e.g. from Fluka)
5.2 Sulfuric acid 50% (e.g. from Bernd Kraft GmbH)
5.3 De-ionized water, HPLC grade (e.g. milli-Q water)
5.4 Acetonitrile (e.g. from Sigma-Aldrich, Chromasolv)
5.5 Sodium sulfate, anhydrous, ultra-pure (e.g. from Merck)
5.6 2-Bromo-2-nitropropane-1,3-diol of known content as a standard (e.g. Merck)
5.7 Sodium bromide of known content as a reference (e.g. Fluka)
5.8 Tris(hydroxymethyl)nitromethane of known content as a reference standard (e.g. from Fluka)
5.9 2-Methyl-2-nitropropane-1,3-diol of known content as a reference standard (e.g. from Acros)
5.10 2-Nitroethanol of known content as a reference standard (e.g. from Aldrich)

6 Apparatus

6.1 HPLC system e.g. Agilent 1100 configured with auto-sampler, gradient pump, column heater, and variable wavelength UV detector
6.1.1.1 Guard column: CC 8/4 NH_2, e.g. packed with Nucleosil 100-5 NH_2 (from Macherey-Nagel)
6.1.2 Separating columns: Stainless steel column (150 x 4.6 mm), e.g. packed with Fluofix 120N, 5 µm from Wako
Stainless steel column (150 x 4 mm), e.g. packed with Aquasil C18, 5 µm from Thermo
6.2 Electronic integrator (e.g. ATLAS from Thermo)
6.3 Standard labware (all glassware must be amber-colored).

7 Procedure

7.1 Solvent for samples and standards

7.1.1 Stabilizer solution
Dissolve approx. 1.25 g of 3-acetamidophenol in acetonitrile in an ultrasonic bath and make up to 25 ml

7.1.2 Diluent
Add 1 ml of 50% sulfuric acid to 1 l of eluent A.

7.2 Preparation of sample

Weigh two portions each of approx. 200 mg, to the nearest 0.01 mg, of the sample into separate 50-ml volumetric flasks and add 0.5 ml of stabilizer solution. Allow to dissolve

Test Method PM/01645/02ee

Assay for bronopol and bronopol impurities by HPLC

and make up with diluent. Vortex for 10 min and inject aliquots into the system to assay for impurities. For the bronopol assay, dilute 5 ml of each of these solutions to 50 ml using the diluent.

7.3 Preparation of calibration solutions:
Weigh approx. 80 mg, to the nearest 0.01 mg, of each of the reference standards (except for bronopol) into separate 100-ml volumetric flasks. Prepare the calibration solutions by diluting the material with the diluent. The calibration solutions cover the anticipated contents of impurities in the samples (in this case from approx. 0.04 to 4 mg/100 ml). The stock solutions of the impurities can be used to prepare calibration solutions which contain all impurities.
Construct two calibration curves for bronopol: in the range from approx. 25 to 50 mg/100 ml for the quantitation of the major component and from approx. 0.04 to 4 mg/100 ml for unknown impurities.
This is achieved by weighing approx. 200 mg, accurately weighed to within 0.01 mg, into a 50-ml volumetric flask. To the bronopol standard only, add 0.5 ml of the stabilizer solution prior to diluting the material with the diluent.
To construct the calibration curves, use at least two sample masses and a minimum of four concentrations prepared therefrom.

7.4 Chromatographic parameters

Guard column: CC 8/4 NH_2, e.g. packed with Nucleosil 100-5 NH_2 from M&N
Analytical columns: Stainless steel column (150 x 4.6 mm), e.g. packed with Fluofix 120N, 5 µm from Wako
Stainless steel column (150 x 4 mm), e.g. packed with Aquasil C18, 5 µm from Thermo
Eluent: A: 5 mM Na_2SO_4
B: 500 ml 5 mM Na_2SO_4 and 500 ml of acetonitrile

Gradient:

Time (min)	0	20	25	40	42	55
% A	100	100	70	70	100	inj
% B	0	0	30	30	0	

Flow rate: 1.0 ml/min
Injection volume: 10 µl (adapted to system sensitivity, as appropriate)
Temperature: 25°C
Detection: 214 nm

7.5 Injection sequence

Usually the solutions are assayed in the sequence below:
- System suitability solutions
- Calibration solutions (by ascending concentrations)

- Sample solutions

If required, blanks can be run in-between.

8 Special system suitability

8.1 Solvent is injected.
Acceptance criteria:
No peak ≥ S/N 10 must elute at the retention times of the components to be quantitated.

8.2 A calibration solution from 7.3 (approx. 1 mg/ 100 ml) is injected.
Resolution between tris(hydroxymethyl)nitromethane and 2-nitroethanol is established in accordance with Ph. Eur.

Acceptance criteria:
Resolution must be ≥2.0.
The two impurities must elute between 4 and 6 minutes. The times may be adjusted by varying the flow rate (± 20%).

9 Calculation

9.1 Calibration factor

$$CF = \frac{AC}{\beta[C]} \frac{mVs * 50\,ml}{mg}$$

where:

CF = calibration factor

AC = peak area of component i [mV x s]

ß(C) = calculated concentration of component i [mg/50 ml]

9.2 Samples:

Quantitation is done by internal standardization

$$w(S) = \frac{PA}{RF * \beta[S]} * 100$$

where:

RF= response factor as established by linear regression

w(S) = mass fraction of component i [g/100g]

PA = peak area of component i [mV . s]

ß(S) = concentration of sample solution [mg/50 ml]

If using Atlas as the software program, the response factor is determined by regression analysis.
Unknown components are quantitated using the bronopol response factor.

10 Uncertainty of measurement

The uncertainty of measurement (UM) is estimated from the standard deviation of the measured values (see validation report).

$$S=\sqrt{\frac{Sum(x-\varnothing)^2}{(n-1)}}$$

where

s = standard deviation
x = measured value
Ø = mean
n = number of measured values

To calculate UM:

UM = 2.83 * s

Thus, the uncertainty of measurement of the method is approx. ± 0.6 % for the major component.
With tris(hydroxymethyl)nitromethane and 2-methyl-2-nitropropane-1,3-diol the UM is ± 1.7% and 2.5%, respectively, at contents of approx. 0.05 g/100g. The measurement uncertainty with sodium bromide is derived therefrom and estimated at < ±3%.

11 Comments

11.1 The experimental work was done by Ms Röschel, GKA/C.
11.2 The test method was validated under job number 09L00001.
11.3 The method has not been tested for 'stability indicating factors'.
11.4 This method replaces PM/00514.

12 Change history

12.1 Author was changed
12.2 General remarks on system suitability tests (hints on laboratory SOP) were given up
12.3 Editorial changes

13 Bibliography

None

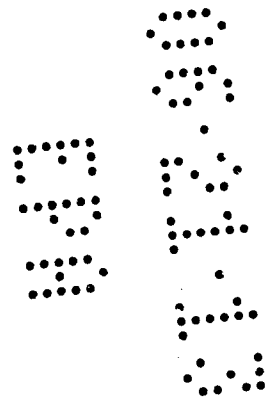
Pages 12-13 - *Access to FIFRA health and safety data (assay results) is restricted under FIFRA section 10(g)*

MYACIDE AS, EPA Registration No. 33753-3
BRITISH PHARMACOPEIA (BP)
BP-METHOD 2011

Reference 5-Batch Analysis Report for Myacide AS, EPA Registration No. 33753-3 dated April 9, 2013 for detail explanation for the purpose of this Analytical Method.

The BP Method is a threshold method and is not designed for calculation of exact concentration of substances. The BP-Method 2011 in addition to PM/01645/02e is used for quantification of Bronopol, related substances and impurities. Due to the co-elution of 2-Methyl-2-nitro-porpane-1,3-diol and the new secondary impurity peak, the BP-Method is used in addition for identification of this co-eluting peak, i.e. differentiation between 2-methyl-2-nitro-propane-1,3-dioland the new secondary impurity. The qualitative information obtained by the BP-Method together with the quantitative information from the PM/01645/02e method, will allow an analysis of the new secondary impurity to ensure that the levels are below 0.1%.

With the combination of PM/01645/02e (for analysis of bronopol and all impurities) and the BP-Method (for identification of the new secondary peak), an analysis regimen is available which allows proper confirmation of the quality profile of Myacide AS in accordance with set specifications.



Limits:
- *impurities A, B, C:* for each impurity, maximum 0.4 per cent of the area of the principal peak;
- *total:* maximum 1 per cent of the area of the principal peak;
- *disregard limit:* 0.1 per cent of the area of the principal peak.

Heavy metals (*2.4.8*)
Maximum 20 ppm.
1.0 g complies with test C. Prepare the reference solution using 2 ml of *lead standard solution (10 ppm Pb) R.*

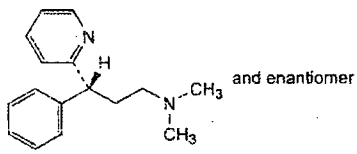
Loss on drying (*2.2.32*)
Maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

Sulphated ash (*2.4.14*)
Maximum 0.1 per cent, determined on 1.0 g.

ASSAY
Dissolve 0.260 g in 50 ml of *anhydrous acetic acid R.* Titrate with *0.1 M perchloric acid,* determining the end-point potentiometrically (2.2.20).
1 ml of *0.1 M perchloric acid* is equivalent to 21.77 mg of $C_{20}H_{23}BrN_2O_4$.

STORAGE
Protected from light.

IMPURITIES
Specified impurities A, B, C.
A. chlorphenamine,
B. dexchlorpheniramine,

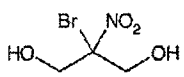


and enantiomer

C. (3*RS*)-*N*,*N*-dimethyl-3-phenyl-3-(pyridin-2-yl)propan-1-amine (pheniramine).

Ph Eur

Bronopol



$C_3H_6BrNO_4$ 200.0 *52-51-7*

Action and use
Antibacterial preservative.

DEFINITION
Bronopol is 2-bromo-2-nitropropane-1,3-diol. It contains not less than 99.0% and not more than 101.0% of $C_3H_6BrNO_4$, calculated with reference to the anhydrous substance.

CHARACTERISTICS
White or almost white crystals or crystalline powder.
Freely soluble in *water* and in *ethanol (96%)*; slightly soluble in *glycerol* and in *liquid paraffin.*

IDENTIFICATION
A. The *infrared absorption spectrum,* Appendix II A, is concordant with the *reference spectrum* of bronopol *(RS 031).*
B. Dissolve 0.1 g in 10 ml of *water,* add 10 ml of 7.5M *sodium hydroxide* and, carefully with constant stirring and cooling, 0.5 g of *nickel-aluminium alloy.* Allow the reaction to subside, filter and carefully neutralise with *nitric acid.* The resulting solution yields reaction A characteristic of *bromides,* Appendix VI.
C. *Melting point,* after drying over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa, about 130°, Appendix V A.

TESTS
Acidity or alkalinity
pH of a 1% w/v solution, 5.0 to 7.0, Appendix V L.

Related substances
Carry out the method for *liquid chromatography,* Appendix III D, using the following solutions in the mobile phase.
(1) 0.2% w/v of the substance being examined.
(2) Dilute a volume of solution (1) to produce a solution containing 0.0002% w/v of the substance being examined.
(3) 0.001% w/v each of *2-methyl-2-nitropropan-1,3-diol* and *tris(hydroxymethyl)nitromethane.*
(4) 0.0002% w/v each of 2-methyl-2-nitropropane-1,3-diol, 2-nitroethanol, sodium bromide and tris(hydroxymethyl)nitromethane and 0.2% w/v of the substance being examined.

CHROMATOGRAPHIC CONDITIONS
(a) Use a stainless steel column (15 cm × 4.6 mm) packed with *octadecylsilyl silica gel for chromatography* (5 µm) (Phenomenex Luna C18 (2) is suitable).
(b) Use isocratic elution and the mobile phase described below.
(c) Use a flow rate of 1 ml per minute.
(d) Use a column temperature of 35°.
(e) Use a detection wavelength of 214 nm.
(f) Inject 20 µl of each solution.
(g) For solution (1) allow the chromatography to proceed for at least 3 times the retention time of the principal peak.

MOBILE PHASE
1 volume of a 10% v/v solution of *orthophosphoric acid,* 10 volumes of *acetonitrile* and 189 volumes of *water,* adjust the pH to 3.0 using 2M *sodium hydroxide.*

SYSTEM SUITABILITY
The test is not valid unless, in the chromatogram obtained with solution (4):
the *resolution factor* between the peaks due to sodium bromide and tris(hydroxymethyl)nitromethane is at least 1.0;
the *resolution factor* between the peaks due to tris(hydroxymethyl)nitromethane and 2-nitroethanol is at least 1.5.

LIMITS
In the chromatogram obtained with solution (1):
the area of any peak corresponding to 2-methyl-2-nitropropane-1,3-diol and tris(hydroxymethyl)nitromethane are not greater than the area of the corresponding peaks in the chromatogram obtained with solution (3) (0.5% of each);
the area of any other *secondary peak* is not greater than the area of the principal peak in the chromatogram obtained with solution (2) (0.1%).

Sulphated ash
Not more than 0.1%, Appendix IX A.

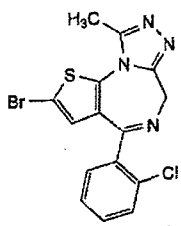
Water
Not more than 0.5% w/w, Appendix IX C, Method I B. Use 5 g.

ASSAY
In a flask fitted with a reflux condenser dissolve 0.4 g in 15 ml of *water* and add 15 ml of *7.5M sodium hydroxide*. Slowly, with caution, add 2 g of *nickel-aluminium alloy* through the reflux condenser, agitating the flask whilst cooling under running water. Allow the mixture to stand for 10 minutes and boil for 1 hour. Cool and filter under reduced pressure, washing the condenser, flask and residue with 150 ml of *water*. Combine the filtrate and washings, add 25 ml of *nitric acid* and 40 ml of *0.1M silver nitrate VS*, shake vigorously and titrate with *0.1M ammonium thiocyanate VS* using *ammonium iron(III) sulphate solution R2* as indicator. Repeat the operation without the substance being examined. The difference between the titrations represents the amount of silver nitrate required. Each ml of *0.1M silver nitrate VS* is equivalent to 20.00 mg of $C_3H_6BrNO_4$.

STORAGE
Bronopol should be protected from light.

Brotizolam

(Ph Eur monograph 2197)



$C_{15}H_{10}BrClN_4S$ 393.7 *57801-81-7*

Action and use
Benzodiazepine.

Ph Eur

DEFINITION
2-Bromo-4-(2-chlorophenyl)-9-methyl-6*H*-thieno-[3,2-*f*][1,2,4]-triazolo[4,3-*a*][1,4]diazepine.

Content
99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS
Appearance
White or yellowish powder.

Solubility
Practically insoluble in water, sparingly soluble or slightly soluble in methanol, slightly soluble in ethanol (96 per cent).

IDENTIFICATION
Infrared absorption spectrophotometry (*2.2.24*).
Comparison *brotizolam CRS*.

TESTS
Related substances
Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

Test solution Dissolve 50.0 mg of the substance to be examined in *acetonitrile R* and dilute to 50.0 ml with the same solvent.

Reference solution (a) Dilute 1.0 ml of the test solution to 100.0 ml of *acetonitrile R*. Dilute 1.0 ml of this solution to 10.0 ml with *acetonitrile R*.

Reference solution (b) Dissolve 5 mg of the substance to be examined and 5 mg of *brotizolam impurity B CRS* in 50 ml of *acetonitrile R*. Dilute 2 ml of this solution to 20 ml with *acetonitrile R*.

Column:
- *size:* l = 0.15 m, Ø = 4.6 mm;
- *stationary phase: octylsilyl silica gel for chromatography R* (5 µm);
- *temperature:* 40 °C.

Mobile phase:
- *mobile phase A*: 2 g/l solution of *sodium heptanesulphonate monohydrate R;*
- *mobile phase B*: mix 25 volumes of a 2 g/l solution of *sodium heptanesulphonate R* and 75 volumes of *acetonitrile R;*

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 4	63	37
4 - 15	63 → 12	37 → 88
15 - 16	12 → 63	88 → 37
16 - 20	63	37

Flow rate 2.0 ml/min.

Detection Spectrophotometer at 242 nm.

Injection 5 µl.

Relative retention With reference to brotizolam (retention time = about 7.4 min): impurity A = about 0.5; impurity B = about 0.9.

System suitability Reference solution (b):
- — *resolution:* minimum 5.0 between the peaks due to impurity B and brotizolam.

Limits:
- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Chlorides (*2.4.4*)
Maximum 100 ppm.
Dissolve 0.67 g in 20.0 ml of *methanol R*, mix and filter.

Loss on drying (*2.2.32*)
Maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

Sulphated ash (*2.4.14*)
Maximum 0.1 per cent, determined on 1.0 g.